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(57) Abstract

A novel method for detecting chitin, and for diagnosing fungal infections (including yeast infections), with a chitinase or other chitin-specific binding protein. This method allows the convenient, broad spectrum diagnosis of fungal infections in tissue samples, body fluids, and other samples. Fungal infections are a particular problem in immunocompromised hosts such as AIDS patients, where they can cause opportunistic infections. This invention overcomes difficulties experienced by prior methods of diagnosing fungal infections.

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DIAGNOSIS OF FUNGAL INFECTIONS WITH A CHITINASE

TECHNICAL FIELD

This invention pertains to the diagnosis of fungal infections, particularly to the diagnosis of fungal infections with a chitinase.

BACKGROUND ART

Fungal infections are a major problem today, particularly in immunocompromised hosts such as acquired immune deficiency syndrome (AIDS) patients or patients receiving a bone marrow transplant. Several million people worldwide are currently infected with the human immunodeficiency virus (HIV). HIV, a retrovirus, causes AIDS. AIDS is characterized by profound derangement in cell-mediated immunity, leading to multiple opportunistic infections and otherwise rare neoplasms.

Opportunistic diseases are the predominant direct causes of morbidity and mortality in AIDS patients. The Centers for Disease Control and the World Health Organization recognized the following fungal infections as important "indicator" diseases in their 1988 definition of AIDS: candidosis of the esophagus, trachea, bronchi, or lungs; and meningeal cryptococcosis. In the presence of laboratory evidence of HIV infection, disseminated coccidioidomycosis and histoplasmosis are also considered indicative of AIDS. It has been estimated that 58-81% of all AIDS patients contract a fungal infection at some time during the course of an AIDS infection, and that 10-20% of AIDS deaths are a direct consequence of fungal infections. Major mycoses related to AIDS include candidosis, cryptococcosis (yeasts), histoplasmosis, and coccidioidomycosis (dimorphic fungi). Deep, severe, but relatively rare mycoses related to AIDS include penicilliosis, blastomycosis, paracoccidioidomycosis, sporotrichosis, aspergillosis, mucormycosis, various yeast infections, and nocardiosis. Cutaneous fungal infections related to AIDS include seborrheic dermatitis, dermatophytosis, trichosporonosis, and alternariosis.

Aspergillosis, although less common in AIDS patients, is a common fungal infection in other immunodepressed patients, such as bone marrow transplant patients, and can occur at a rate as high as 70% in patients with leukemia after 30 days of neutropenia.

In addition to the pathological damage directly caused by fungal infections, fungal antigens may also act as a T-cell suppressor cofactor in the development of AIDS. While small amounts of fungal antigen can stimulate the immune response, an excess of antigen may have an adverse effect on cell-mediated immunity. Circulating fungal antigens, such as mannan in candidosis, and glucuronoxylomannan in cryptococcosis, may be present in excess in acute fungal infections. Candidal antigens in particular may be important cofactors in AIDS. It is imperative that treatment be undertaken rapidly and efficiently before these conditions lead to invasive forms.

Despite their prevalence, systemic fungal infections are difficult to diagnose in living AIDS patients. Unfortunately, autopsy is often the only available route to diagnose fungal infections. AIDS patients with fungal infections may have nonspecific symptoms for long periods of time. It has been difficult to establish definitive diagnoses from patients' body fluids. Histologic identification of organisms requires invasive procedures, with possible attending complications. Isolation of the organisms in blood culture, when possible, can sometimes be used for diagnosis. Even so, proper diagnosis is delayed because of the time required to process the specimens and to culture the fungus. This delay alone can result in progressive deterioration. Some AIDS patients with fungal infections respond to appropriate therapy quickly with early diagnosis, although continued lifetime treatment may be necessary due to the abnormal underlying immune system, and the fact that current anti-fungal pharmaceuticals are fungistatic rather than fungicidal.

To improve the care of AIDS patients and other immunocompromised patients, there is an unfilled need for better means for early diagnoses of fungal infections. Prompt implementation of an appropriate antifungal therapy provides a better environment for antiviral chemotherapy. Despite recent advances in anti-fungal therapeutics that show promise in treating many mycoses, there is a continuing need for a rapid, sensitive, accurate, and broad-spectrum fungal diagnostic method.

There is also an unfilled need for better means to diagnose fungal infections in plant tissues, both in growing plants and in harvested crops and foods. Direct economic losses in agriculturally important crops caused by fungal infections cost billions of dollars annually.

Chitin is a class of polymers of N-acetyl-glucosamine (GlcNAc). Chitin and glucan, another polysaccharide, are the major constituents of the cell walls of most fungi.

Many currently available fungal diagnostic methods are designed to detect specific anti-fungal antibodies in body fluids such as blood or serum, for example, anti-*Candida albicans*, anti-*Cryptococcus*, anti-*Histoplasma*, anti-*Blastomyces*, anti-*Aspergillus* and anti-*Coccidioidomyces*. These antibody-detection tests include immunodiffusion, latex antibody agglutination, complement fixation, and ELISA.

Molecular Probes, Inc. markets a metabolic stain for live fungi; this stain is not useful, however, for staining fungi in fixed histology specimens.

Other methods are available for the selective histologic identification of fungal organisms in tissue specimens, but each of these methods has disadvantages. These methods generally have a broader sensitivity than the antibody detection methods, meaning that they can recognize more than one species of fungi. In addition to their individual disadvantages, a common disadvantage of most existing methods is that they are difficult to apply to samples of body fluids, because proper sample fixation can be difficult.

Grocott methenamine silver nitrate (GMS) staining is by far the most common currently used method in the pathology laboratory. GMS stains polysaccharides in most fungal organisms, creating a contrasting image between the fungus and the host tissue. The stain is not as effective when it is used in cytospin or other body fluid samples. GMS staining can be non-specific, due to its indiscriminate recognition of connective tissue polysaccharides (e.g., glycosaminoglycans and mucin).

Other histochemical stains for fungal organisms include calcofluor/cellufluor, India Ink, lectin label, and Rylus BSU.

Calcofluor/Cellufluor: This method uses calcofluor/cellufluor, which fluoresces under ultraviolet light, to bind to the chitin of fungal organisms. This method inherently relies on fluorescence microscopy, limiting its use in small clinics. Calcofluor/cellufluor is not specific to chitin, as it also labels several other polysaccharides, including cellulose fibers, which are common contaminants in skin samples.

India Ink: This method uses India Ink to detect capsulated organisms such as *Cryptococci*. The method is limited because many fungal cells lack such a capsule.

Lectin label: Lectins having chitin-binding properties (e.g., wheat germ agglutinin) have been used to stain fungi. However, interferences from non-specific staining of other carbohydrates have been a problem.

Rylus BSU: This method also involves staining the chitinous cell walls of fungal organisms with a substance that fluoresces under ultraviolet light. This method also inherently relies on fluorescence microscopy, limiting its use in small clinics.

Chamberland *et al.*, "Chitinase-Gold Complex Used to Localize Chitin Ultrastructurally in Tomato Root Cells," Histochem. J., Vol. 17, pp 313-321 (1985) discusses the use of a fungal-extracted chitinase conjugated with gold to detect chitin in a *Fusarium oxysporum* infection of tomato root cells. Detection was performed with an electron microscope. See alsc Benhamou, *et al.*, "Attempted Localization of a Substrate for Chitinases in Plant Cells Reveals Abundant N-acetyl-D-glucosamine Residues in Secondary Walls," Biology of the Cell, vol. 67, pp. 341-50 (1989).

M.A. Benjaminson, "Conjugates of Chitinase with Fluorescein Isothiocyanate or Lissamine Rhodamine as Specific Stains for Chitin *In Situ*," Stain Technology, vol. 44, pp. 27-31 (1969) discloses the use of a fluorescent-labelled chitinase as a stain for insect and fungus morphology.

M.A. Benjaminson *et al.*, "Ferritin-labelled Enzyme: a Tool for Electron M.croscopy," Nature, vol. 210, pp. 1275-1276 (1966) discloses the use of a ferritin-labelled chitinase to stain *Aspergillus niger*. Fluorescein isothiocyanate-labelled chitinase is also mentioned.

G. Wagner *et al.*, "Chitin in the Epidermal Cuticle of a Vertebrate (*Paralipophrys trigloides*, Blenniidae, Teleostei)," Experentia, vol. 49, pp. 317-319 (1993) reported finding the presence of chitin in the epidermal cuticle of a bony fish.

There is a continuing need for new methods for diagnosing fungal infections :n samples such as animal and human tissues and fluids.

DISCLOSURE OF THE INVENTION

This invention provides a novel means of using a chitinase or other chitin-specific binding protein for diagnosing infections from fungal organisms, including yeasts, in animal and human tissues or body fluids, as well as in other samples. The novel method is simple, rapid, sensitive, quantitative, and general. A preferred chitinase, isolated and cloned from *Vibrio parahemolyticus* and designated "Chitinase VP1," binds so tightly to chitin that it (and an anti-chitinase antibody) can be used as a histochemical diagnostic probe with great sensitivity to specifically visualize fungal cell walls or yeast bud scars in tissue sections. A filter assay, enzyme-linked system will detect small amounts of fungal cell wall or yeast bud scar materials present in body fluids. The molecularly cloned Chitinase VP1 can be expressed and secreted in *E. coli* at a high level, producing about 30 mg/liter of culture medium.

The term "chitinase" generally describes an enzyme specific for the substrate chitin. Many different types of chitinases occur naturally. For example, chitinases are found in microbes such as *Serratia*, *Vibrio*, and *Streptomyces*.

There are many glucosamine-containing, and N-acetyl-glucosamine-containing, compounds present in a typical animal species. Whether chitin, an N-acetyl-glucosamine-containing polymer, could be specifically identified by its corresponding enzyme from this background was not previously known or suggested. Furthermore, because the biological function of the chitinase enzymes is to degrade chitin, it is an unexpected result that a chitinase can bind to chitin tightly enough and for a long enough period of time, while surviving repeated washings, to serve as an effective stain, or a means of detecting chitin in animals. To the inventors' knowledge, no chitinase has previously been used to detect chitin or fungi (including yeasts) in an animal species or in humans.

This novel method will broadly detect a wide variety of fungal infections (including yeast infections), and may be used quantitatively. This test allows an early and definitive diagnosis of the etiologic fungal organisms causing opportunistic infections, so that implementation of an appropriate antifungal therapy can be initiated promptly. The assay recognizes yeasts by the chitin present in the yeast bud scars.

Chitinases may be used as the basis for an easy-to-use stain for diagnosing fungal infections, including yeast infections, in plant, animal, and human tissues or fluids. (Plant materials may include living tissues, as well as grains, fruits, vegetables, tubers, and other agricultural products.) The diagnostic method is simple, rapid, sensitive, and general. Chitinase specifically stains fungal cell walls or yeast bud scars with high sensitivity. A filter assay, enzyme-linked system will detect small amounts of fungal cell wall or yeast bud scar materials present in body fluids, other fluids, potable water samples, or beverages. An air filter assay, using enzyme-linked or chromogenic systems, will detect small amounts of fungal cell wall or yeast bud scar materials in air filters, which can also be contaminated with fungi and their spores. Likewise, fungal contamination in contact lenses or other prostheses can be assayed.

This assay, analogous to ELISA, can for example use an immobilized chitinase that is placed in contact with a sample. After rinsing unbound sample away, adherent chitin fragments will bind mobile, enzyme-linked chitinase, for example, a chitinase linked to horseradish peroxidase. The unbound enzyme-linked chitinase is washed away, and chitin is detected by reaction of the linked enzyme with a suitable substrate.

Polymeric chitin is produced neither by mammals nor by higher plants, although (as discussed by Wagner *et al.*, cited above) chitin may be present in some fish. Any chitin found in a mammalian host is necessarily of non-mammalian origin, and (other than in the digestive tract) implies the presence of an infection. Natural chitin sources other than fungi include a number of invertebrates; chitin is notably found in the exoskeletons of arthropods. These

potential non-fungal sources of chitin should not significantly interfere with the use of chitinase to identify fungal infections.

BEST MODE FOR CARRYING OUT THE INVENTION

We have cloned the preferred Chitinase VP1 gene from *Vibrio parahemolyticus* into the pKK233 plasmid to create a new plasmid designated pKKA1, which was then transformed into *E. coli* strain JM101. The cloned Chitinase VP1 gene was expressed, and Chitinase VP1 was secreted efficiently into the medium. The enzyme was easily purified by ammonium sulfate precipitation. Its purity exceeded 90%, as determined by SDS-PAGE. The cloned Chitinase VP1 remained active within an unusually wide range of pH (pH 4.5-8.8), salt concentration (as high as 4M NaCl), and temperature (as high as 50° C). Further details regarding the isolation, characterization, cloning, and sequencing of the novel Chitinase VP1 and its gene may be found in the following two manuscripts: Ou *et al.*, "Isolation, Characterization and Molecular Cloning of an Endo-Chitinase from *Vibrio parahemolyticus*," Louisiana State University draft manuscript (1991); and Lo *et al.*, "DNA Sequence and High Expression Level for an Endo-Chitinase from *Vibrio parahemolyticus*," Louisiana State University draft manuscript (1991).

A sample of this transformed *E. coli* strain JM101 with the cloned Chitinase VP1 gene in plasmid pKKA1 was deposited under the Budapest Treaty with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on 29 January 1992, and was assigned ATCC Accession No. 68906. This deposit was made pursuant to a contract between ATCC and the assignee of this patent application, Board of Supervisors of Louisiana State University and Agricultural and Mechanical College. The contract with ATCC provides for the permanent and unrestricted availability of the progeny of this *E. coli* strain to the public on the issuance of the U. S. patent describing and identifying the deposit or the publication or the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of this *E. coli* strain to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto under pertinent statutes and regulations. The assignee of the present application has agreed that if the *E. coli* strain on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same *E. coli* strain.

As used in the claims below, the term "Chitinase VP1" is intended to include not only exact duplicates of this enzyme, but also any enzyme having substantially the same amino acid sequence, and substantially the same chitin-binding activity.

With the use of a label conjugate complex, the presence of intact fungal organisms or fragments of chitinous cell walls can be quantified. It is expected that chitinases and other chitin-

specific binding proteins, especially those conjugated to another enzyme to amplify the signal, will detect fragments of chitinous cell walls or yeast bud scars in circulation, even in cases where the fragments alone would not result in a viable culture for conventional organism isolation. The invasive biopsy required in many immunodepressed hosts for a proper diagnosis may be needed less often through the use of the filtration assay of body fluids in accordance with this invention.

Chitinase or other chitin-binding protein, preferably conjugated to a label, can be used to detect the presence of intact fungal organisms or fragments of chitinous cell walls. Especially if conjugated to an enzyme to amplify the detection signal (analogous to ELISA), chitinase or other chitin-binding protein will detect fragments of chitinous cell walls or yeast bud scars in circulation, even in cases where the fragments alone could not be used to culture the infecting organism. For example, the presence of chitin in etiologic agents of mycoses from tissue specimens may be verified by a system such as chitinase/rabbit anti-chitinase antibody/fluorescent-labeled goat anti-rabbit antibody; or a system such as chitinase directly coupled to fluorescein isothiocyanate (FITC); or an enzyme-linked system such as horseradish peroxidase, alkaline phosphatase, or β-galactosidase directly coupled to chitinase, or an indirect antibody assay.

Chitin is present in the cell walls of most pathogenic fungi during at least one stage of the fungal life cycle, or in yeast is present in bud scars. A chitin-specific stain will therefore be specific for fungi (chitin being generally absent from bacteria and infectious protozoans), and will be a broad spectrum stain for most kinds of fungi. Fortunately, many current treatments for systemic fungal infections, for example amphotericin-B and azoles, are broad-spectrum in effect. A rapid, sensitive, and accurate diagnostic method that provides an early, definitive diagnosis of disease-causing fungal organisms (perhaps without the need of a species-specific classification) will be more beneficial for patients (particularly AIDS patients and bone marrow transplant patients), than other less sensitive or more time-consuming processes that attempt to identify particular species. Morphology of the fungal organisms delineated by a chitinase label generally provides sufficient information to identify the genus of the organisms. Species-specific identification, if needed, can be made at a later opportune time using other conventional methods, such as blood cultures. The course and efficacy of an antifungal treatment can readily be monitored with the novel staining technique.

It may be difficult in some cases to distinguish between normal surface flora and disseminated deep infections in histological samples, smears, and other superficial samples from oral-esophageal fungal infections. A large number of samples from this kind of infection, such as candidosis, will be inspected to establish differences between samples from the *Candida*, and those of normal mucosal flora.

The presence of chitin in etiologic agents of mycoses from animal and human tissue specimens will be verified either by a system of chitinase/rabbit polyclonal or monoclonal anti-chitinase/fluorescent goat anti-rabbit, or by chitinase directly coupled to fluorescein isothiocyanate (FITC). An enzyme-linked approach will use horseradish peroxidase directly coupled to chitinase, or an indirect antibody sandwich.

The high purity of the cloned Chitinase VP1 provided antigen for the production of a specific anti-chitinase polyclonal antibody in rabbit through techniques known in the art. The use of a monoclonal antibody in a probe was therefore unnecessary, although production of monoclonal antibodies through techniques known in the art is, of course, also possible. *Aspergillus niger* grown in bacteriologic media was successfully labelled using the cloned Chitinase VP1 and anti-Chitinase VP1 antibody with anti-rabbit IgG conjugated to FITC.

The system was tested in animal and human tissue sections with known fungal infections already identified by GMS or other tests. Tissue sections from animals with aspergillosis, cryptococcosis, and blastomycosis, as well as a tissue sample from a human AIDS patient with candidosis, were tested with the Chitinase VP1/anti-Chitinase VP1 antibody probe, or with a Chitinase VP1-FITC direct conjugate probe, and examined. *Aspergillus* and *Blastomyces* were positively identified in the presence of appropriate controls. *Cryptococcus neoformans*, which is frequently encapsulated with a thick polysaccharide, appeared to be labeled on a selective basis. In contrast to active organisms, most of the organisms with a thick polysaccharide capsule were poorly stained. Additional tests will determine if the selective staining is due to the lack of chitin in those cell walls, or to the inaccessibility of the probe due to the thick capsule.

The spectrum breadth of the diagnostic probe will be confirmed by identifying chitin in the fungal cell walls or yeast bud scars of the most common opportunistic fungi and true pathogenic fungi in samples from human patients, and in samples from plants and animals of economic significance. The present invention can be used to verify the presence of chitin in these organisms in samples prepared in thin sections. Chitinous fungi will be categorized separately from non-chitinous fungi, if any of the latter are found in this screening. It is expected that the accessibility of chitin may in some cases be partially or totally blocked by the polysaccharide capsule found in some fungi, such as that of *Cryptococcus*. Establishing the extent of this blockage will help to improve the diagnostic system. For example, proteases or polysaccharidases such as a glucanase or mannanase may be used as an adjuvant to uncover chitin in a complex cell wall, to facilitate accessibility to chitinase. Alternatively, a 1-5% periodate solution can be used to digest the polysaccharide capsule. Furthermore, even in a single infection, the biochemical components of *Cryptococci* can vary over the course of the life cycle. Thus at least some

Cryptococci should be accessible to this diagnostic method at any given time, even if the polysaccharide capsule blocks the accessibility of chitin in some of the organisms.

Opportunistic fungal organisms are generally nonpathogenic in hosts with healthy immune systems. The validity of this diagnostic system will be confirmed with a variety of opportunistic fungal organisms, including those most commonly found in AIDS patients. Tissue preparation prior to staining will be conducted in accordance with standard procedures for fixed, paraffin-embedded tissues. The cells will be fixed with either methanol or 4% paraformaldehyde. The specimen will be embedded in paraffin and thin-sectioned. After a series of standard deparaffination procedures with xylene and a series of ethanol solutions at different concentrations, chitinase (100 μl of 1.0 mg/ml solution) and diluted, labelled anti-chitinase (100μl of a 1 to 10 μg/ml solution) will be applied to BSA-pretreated specimens. Each specimen will be washed with PBS between additions of reagents. Endogenous peroxidase in the specimen will be removed if peroxidase conjugate is used as the labeling probe. Several labeling probes will be investigated, to identify an optimal label for these tests. For example, the efficacy of Protein A-Peroxidase or Protein A-FITC conjugate, anti-rabbit IgG-Peroxidase or FITC conjugate, peroxidase-antiperoxidase complex, and avidin-biotin will be evaluated. It may be advantageous to label the chitinase directly with various labels known in the art, to eliminate the need for an anti-chitinase antibody. For example, direct conjugates of chitinase with FITC or with horseradish peroxidase will be examined. Whether the chitin-specific binding protein or its antibody is labelled, detectable labels that may be used are labels known in the art, including a radioactive material, a fluorophore, a dye, an electron-dense compound, or an enzyme. The variety of potential label possibilities broadens the potential applications of this invention. A conventional GMS fungal stain will be used in parallel for comparison.

The diagnostic system will also be validated by testing chitinous materials in suspension, to demonstrate that the system also works in diagnosis with fluid samples of biological origin. A small filter unit (Spin-X, CoStar Co.) equipped with a polyvinylidene difluoride (PVDF, Immobilon, Millipore, USA) membrane pretreated with BSA will be used to retain chitinous materials, specifically swollen chitin in suspension. Other reagents in the diagnostic probe will be applied in the following order: chitinase, anti-chitinase antibody, and Protein A-Peroxidase conjugate. (The use of enzyme in place of FITC will allow quantitative analysis if desired.) After addition of a chromogenic developing solution (e.g., 4-chloro-N-naphthol and H_2O_2, or dyes such as CY5 (Amersham, Inc.)), only units with chitin, chitinase, and anti-chitinase should be positive. It should be feasible to retain chitinous fungal organisms, or their chitinous cell wall or yeast bud scar materials, on the membrane without interference by other proteins present in

body fluids. Subsequently, the retained chitinous materials can be qualitatively detected by the diagnostic probe if desired: chitinase (with or without anti-chitinase antibody), and a chromogenic label conjugate.

The following method will be used to demonstrate further the feasibility of this technique for diagnosis in body fluids. A set of fungal organisms will be grown in suspension. The cells will be mixed with normal human serum, and processed using a small filter unit as the retention matrix. The smallest number of fungal organisms or the smallest amount of fungal cell wall or yeast bud scar materials in 100 μl of serum giving a definitive result will be determined. The contaminated serum will be diluted for easy filtration, and prospective fungal organisms or fragments of cell walls or bud scars trapped by the membrane will be fixed with either methanol or paraformaldehyde. The filter will be washed twice with distilled water to lyse red blood cells when dealing with actual specimens, and then washed three times with PBS plus 0.05% Tween 20, followed by the addition of 100 μl of chitinase (1.0 mg/ml) for 20 min at room temperature. (Digestion at a higher temperature will also be attempted.) After washing the filter with PBS, diluted anti-chitinase antibody (100 μl) will be added to the filter and incubated for 30 min at room temperature. A labeled probe will be added to the filter after washing. Quantitative analysis can be achieved by using an appropriate labeled probe. If peroxidase is used in conjugation, a soluble chromogenic developing solution with ABTS can be used for quantitative analysis. Direct conjugates of chitinase will also be used in this study. To test that the system is specific for fungal organisms, several control groups will be tested as well. These control groups will include serum contaminated with known bacteria, viruses, or protozoa. Alternatively, direct labelling of chitinase with visible dyes will be used for light microscopy and flow detection systems.

For example, fragments of *Aspergillus* cell wall, mycelial fragments obtained by sonication, and whole cells will each be suspended separately, and the suspensions diluted ten or more times. Each resulting solution will be filtered through a 10,000 molecular weight cut-off filter. Chitinase, rabbit anti-chitinase antibody, and horseradish peroxidase-labelled goat anti-rabbit IgG will be applied to the filters (a direct conjugate will also be used), and the catalytic potential of the bound enzyme will be assayed to determine the sensitivity of the assay for cell wall fragments in body fluids.

It has previously been the case that certain deep invasive fungal infections could only be properly diagnosed by histologic examination. It is possible that certain fungal antigens or whole cells might substantially disappear from circulation during certain stages of disease. In such cases it would still be desirable and safer to obtain a definitive diagnosis by testing body fluids if possible, rather than by examining histologic specimens. Comparisons will therefore be made

between the effectiveness of chitinase as a diagnostic probe in samples of body fluids, with results from other diagnostic methods presently in use (including histologic identification if necessary).

Specimens collected from plants and animals will be processed appropriately for histologic identification by embedding and thin sectioning before specific staining procedures, with tissue samples prepared by standard protocols. Also, fluid samples will be processed for in vitro testing in a small filter unit using chitinase as a diagnostic probe. The procedures will be generally as described above. Correlation with other diagnostic procedures will be evaluated in a blind study with several control groups. Specimens from plants and animals with non-fungal infections, bacteremia, viral infection, and other disorders uncomplicated by fungal infections will also be tested as controls. Later, similar tests will be conducted with specimens from human patients, including AIDS patients and bone marrow transplant patients.

It will also be appreciated by those of skill in the art that certain other chitin-specific binding proteins can be used in place of a chitinase, without departing from the spirit of the invention. An example of such a protein is the chitin-adhesion protein of *Vibrio furnissii*; see Yu *et al.*, J. Biol. Chem., vol. 266, pp 24260-24267 (1991) (not admitted to prior art), the entire disclosure of which is incorporated by reference.

Other substantially pure chitinases or other substantially pure chitin-specific binding proteins will work in place of Chitinase VP1. Determining whether a particular chitinase or chitin-specific binding protein will work in this invention is well within the skill of the ordinary worker in the art, following the experimental guidelines given above. As used in the claims below, a "substantially pure" chitinase or chitin-specific binding protein is one in which any contaminants that may be present are in sufficiently low concentrations as to avoid significant interference with the specificity and selectivity of the chitin-binding reaction in diagnosing the presence of chitin or fungi (including yeasts) in a sample; in other words, the concentrations of any contaminants in a "substantially pure" chitinase or chitin-specific binding protein do not significantly affect the frequency of false positive or false negative results. By way of example, the cloned Chitinase VP1, isolated at 90% purity as described above, is considered to be "substantially pure."

What is claimed is:

1. A method for detecting chitin in a sample comprising a human tissue, an animal tissue, a human body fluid, an animal body fluid, a contact lens, a prosthetic device, an air filter, potable water, or a beverage, said method comprising the steps of:

(a) contacting the sample with a substance comprising Chitinase VP1;

(b) analyzing the sample for the presence of Chitinase VP1 bound to chitin.

2. A method as recited in claim 1, wherein said Chitinase VP1 is conjugated to a detectable label.

3. A method as recited in claim 2, wherein the detectable label is selected from the group consisting of radioactive material, fluorophore, dye, an electron-dense compound, and an enzyme.

4. A method as recited in claim 2, wherein the sample comprises an animal tissue or a human tissue.

5. A method as recited in Claim 4, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in fungal cell walls.

6. A method as recited in Claim 4, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in yeast bud scars.

7. A method as recited in claim 2, wherein the sample comprises an animal body fluid or a human body fluid.

8. A method as recited in Claim 7, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in fungal cell walls.

9. A method as recited in Claim 7, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in yeast bud scars.

10. A method as recited in claim 1, additionally comprising the step of quantitatively assaying the sample for Chitinase VP1-chitin complexes as a measure of the amount of chitin or fungi in the sample.

11. A method as recited in claim 1, additionally comprising the step of contacting the sample with at least one reagent comprising an antibody to Chitinase VP1.

12. A method as recited in claim 11, wherein at least one said reagent is conjugated to a detectable label.

13. A method as recited in claim 12, wherein the detectable label is selected from the group consisting of radioactive material, fluorophore, dye, an electron-dense compound, and an enzyme.

14. A method as recited in claim 12, wherein the sample comprises an animal tissue or a human tissue.

15. A method as recited in Claim 14, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in fungal cell walls.

16. A method as recited in Claim 14, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in yeast bud scars.

17. A method as recited in claim 12, wherein the sample comprises an animal body fluid or a human body fluid.

18. A method as recited in Claim 17, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in fungal cell walls.

19. A method as recited in Claim 17, wherein said analyzing step comprises analyzing the sample for the presence of Chitinase VP1 bound to chitin in yeast bud scars.

20. A method as recited in claim 11, additionally comprising the step of quantitatively assaying the sample for Chitinase VP1-chitin complexes as a measure of the amount of chitin or fungi in the sample.

21. A diagnostic test kit for diagnosing the presence of chitin or fungi in a sample comprising a human tissue, an animal tissue, a human body fluid, an animal body fluid, a contact lens, a prosthetic device, an air filter, potable water, or a beverage, said kit comprising Chitinase VP1, and instructions for:

(a) contacting the sample with said Chitinase VP1; and

(b) analyzing the sample for the presence of Chitinase VP1 bound to chitin.

22. A diagnostic test kit as recited in claim 21, additionally comprising an antibody to Chitinase VP1.

23. A method for detecting chitin in a sample comprising a human tissue, an animal tissue, a human body fluid, an animal body fluid, a contact lens, a prosthetic device, an air filter, potable water, or a beverage, said method comprising the steps of:

(a) contacting the sample with a substance comprising a substantially pure chitin-specific binding protein;

(b) analyzing the sample for the presence of the chitin-specific binding protein bound to chitin.

24. A method as recited in claim 23, wherein said substantially pure chitin-specific binding protein comprises a substantially pure chitinase.

25. A method as recited in claim 23, wherein said substance is conjugated to a detectable label.

26. A method as recited in claim 25, wherein the detectable label is selected from the group consisting of radioactive material, fluorophore, dye, an electron-dense compound, and an enzyme.

27. A method as recited in claim 25, wherein the sample comprises an animal tissue or a human tissue.

28. A method as recited in Claim 27, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in fungal cell walls.

29. A method as recited in Claim 27, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in yeast bud scars.

30. A method as recited in claim 25, wherein the sample comprises an animal body fluid or a human body fluid.

31. A method as recited in Claim 30, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in fungal cell walls.

32. A method as recited in Claim 30, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in yeast bud scars.

33. A method as recited in claim 23, additionally comprising the step of quantitatively assaying the sample for complexes of chitin and the chitin-specific binding protein as a measure of the amount of chitin or fungi in the sample.

34. A method as recited in claim 23, additionally comprising the step of contacting the sample with at least one reagent comprising an antibody to said chitin-specific binding protein.

35. A method as recited in claim 34, wherein said substantially pure chitin-specific binding protein comprises a substantially pure chitinase, and wherein said antibody comprises an antibody to said chitinase.

36. A method as recited in claim 34, wherein at least one said reagent is conjugated to a detectable label.

37. A method as recited in claim 36, wherein the detectable label is selected from the group consisting of radioactive material, fluorophore, dye, an electron-dense compound, and an enzyme.

38. A method as recited in claim 36, wherein the sample comprises an animal tissue or a human tissue.

39. A method as recited in Claim 38, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in fungal cell walls.

40. A method as recited in Claim 38, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in yeast bud scars.

41. A method as recited in claim 36, wherein the sample comprises an animal body fluid or a human body fluid.

42. A method as recited in Claim 41, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in fungal cell walls.

43. A method as recited in Claim 41, wherein said analyzing step comprises analyzing the sample for the presence of the chitin-specific binding protein bound to chitin in yeast bud scars.

44. A method as recited in claim 34, additionally comprising the step of quantitatively assaying the sample for complexes of chitin and the chitin-specific binding protein as a measure of the amount of chitin or fungi in the sample.

45. A diagnostic test kit for diagnosing the presence of chitin or fungi in a sample comprising a human tissue, an animal tissue, a human body fluid, an animal body fluid, a contact lens, a prosthetic device, an air filter, potable water, or a beverage, said kit comprising a substantially pure chitin-specific binding protein, and instructions for:

(a) contacting the sample with the chitin-specific binding protein; and

(b) analyzing the sample for the presence of the chitin-specific binding protein bound to chitin.

46. A diagnostic test kit as recited in claim 45, wherein said substantially pure chitin-specific binding protein comprises a substantially pure chitinase.

47. A diagnostic test kit as recited in claim 45, additionally comprising an antibody to said chitin-specific binding protein.

48. A diagnostic test kit as recited in claim 47, wherein said substantially pure chitin-specific binding protein comprises a substantially pure chitinase, and wherein said antibody comprises an antibody to said chitinase.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11738

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/569
US CL : 435/7.31
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.31, 252.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, DIALINDEX:SF ALLSCIENCE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,352,607 A (LAINE ET AL) 04 October 1994, see entire document, especially column 3, lines 9-32 and column 6, lines 41-51.	1-48
X -- Y	WALKER et al. Immunohistochemical detection of chitin in pneumocystis-carinii. Laboratory Investigation. 04-09 March 1990, Vol. 62(1), page 104A, #616, see whole document.	23-45 ----- 45-48
X -- Y	WALKER et al. Labelling of invasive opportunistic fungi immunocytochemical detection of chitin. Laboratory Investigation. 17-22 March 1991, page 90A, #529, see whole document.	23-48 ----- 45-48
Y	WO 92/17786 A (SRI INT) 30 March 1992, page 1, abstract, see whole document.	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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29 AUGUST 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11738

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ILYINA et al. One-step isolation of a chitinase by affinity chromatography of the chitnolytic enzyme complex produced by Streptomyces kurssanovii. Biotechnology Applied Biochemistry. 1994, Vol. 19, pages 199-207, especially pages 199-200.	1-48